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Survival of *Streptococcus agalactiae* from frozen fish following natural and experimental infections

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Abstract

We evaluated the survival of *Streptococcus agalactiae* from naturally infected wild mullet (*Liza klunzingeri*) and experimentally infected Nile tilapia (*Oreochromis niloticus*) frozen at −20 and −70 °C, respectively, for an extended period of time. The brain, eye, head kidney, and intestine of individually frozen wild mullet ($N=22$), culture positive for *S. agalactiae* from fresh tissues following an *S. agalactiae* epizootic in Kuwait Bay in 2001, were re-sampled after 9 months. Nares, eye, head kidney and intestines, not previously sampled, were also evaluated. Tilapia were inoculated with either 5.6×10^2 colony forming units CFU/fish or 4.5×10^6 CFU/fish and *S. agalactiae* survival assessed from frozen tissues after 7, 14, 30, or 180 days. *S. agalactiae* was recovered from nare, brain, eye, and head kidney of 100% of the frozen mullet after 9 months post-freezing. The nare, brain, and head kidney of 100% of the experimentally infected tilapia were culture positive at 7, 14, 30, and 180 days post-freezing. The use of frozen fish may prove to be a useful alternative to fresh fish for recovering pathogenic streptococci in instances when fresh fish diagnostic analysis are unavailable or impractical. Furthermore, archived frozen fish can be used for retrospective microbiological analyses of streptococcal infection from multiple or different tissues not originally sampled.
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Keywords: *Streptococcus agalactiae*; Frozen fish; Infection

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1. Introduction

The duration of bacterial viability in frozen fish tissues or the length of time tissues can remain frozen and still yield viable bacteria upon partial thawing is an area warranting further study. Most studies have concentrated on clinical isolates or clinical food borne pathogens of epizootic concern in frozen products. Cooling or freezing of clinical specimen collections have been recognized as extending the viability of pyogenic cocci (Stuart, 1959). Murray (1998) indicated that clinical specimen collections harboring Gram positive cocci can be stored at −4 °C for prolonged times. Facklam and Washington (1991) indicated survival of Gram positive cocci for 1 to 2 years frozen in blood at −70 °C and over 20 years if they are lyophilized. In one of the few studies of bacterial viability in frozen fish, Brady and Vinitnantharat (1990) found viable, pathogenic, Gram negative bacteria could be recovered from channel catfish (*Ictalurus punctatus*) frozen at −20 °C for over 20 days.

Due to the sudden occurrence of streptococcal disease outbreaks in remote locations worldwide, there is the need for fish collection and storage methodologies that can be employed by environmental agency personnel and aquaculturists in the absence of trained fish pathologists. Microbiological samples collected from fresh, naturally infected mullet (*Liza klunzingeri*) using the Bacti-Swab™ NPG collection and transport system, with media ampules intact and in transit for 4 days before laboratory receipt and enrichment technique, yielded viability of *Streptococcus agalactiae* from 82.1% of diseased mullet (Evans et al., 2002a). Using this same methodology in experimental infectivity studies, Evans et al. (2002b) examined a collection and dry transport system for retaining viability of streptococcal organisms over varying time periods and the enrichment technique for re-isolation of the organisms. *S. agalactiae* collected from infected tilapia remained viable for 4 to 10 days following collection and enrichment. Although live, moribund fish are recognized as ideal for the isolation and determination of pathogenic bacteria, frozen fish may be suitable for bacterial analysis provided the samples remain frozen in transit and are delivered to the diagnostic laboratory within an acceptable time period (Brady and Vinitnantharat, 1990). Previously, Evans et al. (2002a) recovered viable *S. agalactiae* from mullet and sea bream (*Sparus auratus* L.) frozen for 38 days using the enrichment technique. In this study, we examined the duration of *S. agalactiae* survival in frozen fish organs including the nares of two species of fish following natural or experimental infection as an alternative means of collection and storage until microbiological analyses are performed. Furthermore, we examined the use of the enrichment technique to isolate or re-isolate *S. agalactiae* from frozen fish.

2. Materials and methods

2.1. Kuwait epizootic fish

Live, moribund mullet were collected from Kuwait Bay in early September 2001 and microbiological samples were obtained and analyzed as described by Evans et al. (2002a). After necropsy, fish were individually bagged, placed on ice, transported back to the

Kuwait Environmental Public Authority (EPA) Laboratory and frozen at −20 °C. The fish were packed on wet ice in coolers and hand carried during transport to the University of Maryland, Center for Environmental Studies, USA within 6 days of collection. Frozen fish were retained at −20 °C for 9 months and later transported on ice to USDA, ARS, Aquatic Animal Health Research Laboratory, Chestertown, MD in late May 2002 and stored at −70 °C. Twenty-two *S. agalactiae* infected frozen mullet were re-sampled on May 29, 2002 for the presence of *S. agalactiae* in organs originally sampled, in nares of all fish, and the eye, head kidney, and intestines of fish not sampled at the time of collection.

2.2. Experimental fish

Nile tilapia, *Oreochromis niloticus*, of mixed sexes were reared from the stock of the USDA, ARS, Aquatic Animal Health Research Laboratory, Auburn, AL. The fish (mean weight = 68.6 g) were maintained in flow-through 57 l glass tanks supplied with an air stone, and 0.5 l/min of de-chlorinated water. The fish were maintained in a photoperiod of 12 L:12 D. The fish were fed once a day with Aquamax Grower 400 (Brentwood, MO) to satiation. The dissolved oxygen (DO) and temperature were measured using a YSI 85 oxygen, conductivity, salinity, and temperature meter (Yellow Springs Instrument, Yellow Springs, OH). The pH, hardness, ammonia, and nitrites were determined using the Fresh Water Aquaculture Kit Model AG-2 (LaMotte, Chestertown, MD). The average water quality conditions were within acceptable ranges for tilapia.

2.3. Tilapia infectivity

Tilapia ($N = 60$) were separated into three groups of 20 fish each (two treatment and one control group) and placed in aerated 57 l tanks at 32 °C. Prior to initiation of infectivity trials, brain, and head kidneys from five tilapia from laboratory stock were cultured for the presence of *S. agalactiae*. All fish were culture negative. *S. agalactiae*, designated ARS-KU-11B (National Agricultural Research Collection NRRL B-30607), was obtained from the brain of a diseased mullet during the Kuwait epizootic. The inocula were prepared by streaking this isolate on five sheep blood agar (SBA) (Remel, Lenexa, KS) plates and allowed to grow in an environment of air for 24 h at 35 °C. Following incubation, the cells were harvested utilizing sterile wooden scrapers. The cells were transferred into two 9 ml aliquots of sterile tryptic soy broth (TSB; Difco Laboratories, Detroit, MI) and adjusted to a MacFarland standard number 5. The inocula were mixed via inversion and allowed to stand for 2 min. The bacterial inocula were diluted to 4.5×10^7 and 5.5×10^3 colony forming units CFU/ml in sterile TSB and placed in an ice bath. The actual number of CFU/ml was determined using a spiral autoplater and Qcount (Spiral Biotech, Norwood, MA).

Twenty fish each received 0.1 ml of 4.5×10^7 or 5.5×10^3 CFU/ml by intraperitoneal (IP) injection. Twenty control fish each received 0.1 ml of sterile TSB. At 24 h, all treatment tilapia, irrespective of condition (dead, morbid, and asymptomatic), and control tilapia were removed from aquaria, separated by treatment into four groups of five fish, and frozen at −70 °C. The tilapia were frozen for 7, 14, 30, or 180 days.

2.4. Bacterial retrieval and identification

After approximately 9 months of frozen storage (Kuwait mullet) or following four frozen time periods (tilapia), fish were partially thawed at ambient temperature (25–26 °C) for 2–3 h. Mullet brain, eye, intestine, and head kidney culture positive for *S. agalactiae* at initial sampling were re-swabbed using a sterile mini-tip swab (Bacti-Swab™ NPG, Remel). In addition, the posterior nare, eye, head kidney, and intestine of any mullet, not previously sampled, were also swabbed. The nare, brain, intestine, and head kidney of five partially thawed tilapia from each time period were sampled as above. The swabs were placed into 5 ml of TSB supplemented with three to four drops of defibrinated sheep blood and incubated at 30 °C for 3–4 h. Following enrichment, the bacterial cultures were streaked onto SBA and agar stabbed in three areas with a sterile inoculating loop for colony differentiation and hemolysis testing, respectively. The plates were incubated in air for 24 h at 35 °C and assessed for growth and β-hemolysis. Single colonies exhibiting *S. agalactiae* phenotypic characteristics were selected for Gram staining, oxidase, catalase testing, and Biolog (Haywood, CA) microbial identification.

3. Results

3.1. Kuwait epizootic fish

Mullet organs culture positive for *S. agalactiae* at initial sampling were also positive when re-sampled after 9 months post-freezing. One hundred percent of the frozen mullet nare, brain, eye, and head kidney, not previously sampled for microbiological analyses,

Table 1
Percentage of mullet organs culture positive for *S. agalactiae* from the fresh (9/2–9/3/01) and from the frozen organ sampling after 9 months at −20 °C (5/29/02)[a]

Percentage of organs culture positive for *S. agalactiae*				
Organ sampled	Fresh organs sampled on 9/2–9/3/01		Frozen organs sampled on 5/29/02	
	Number of organs sampled	% Positive (# positive)	Number of organs sampled[b]	% Positive (# positive)
Nare	NS[c]	–	22	100 (22)
Brain	22	63.6 (14)	14	100 (14)
Eye	6	33.3 (2)	12	100 (12)
Head kidney	13	30.8 (4)	9	100 (9)
Intestine	3	33.3 (1)	20	95 (19)
Total	44	47.7 (21)	77	98.7 (76)

[a] Not all mullet sampled in Kuwait were received frozen for these analyses ($N=22$). All microbiological analyses performed in the USA. A total of 81.8% (18/22) of the fresh fish were culture positive and 18.2% (4/18) were culture negative for *S. agalactiae*.

[b] Of the 22 frozen fish received, organs testing positive for *S. agalactiae* from fresh fish and organs not originally sampled (nare = 22; eye = 10; head kidney = 5; intestine = 19) on 9/2-9/3/01 from culture positive fish were cultured.

[c] No samples were taken.

Table 2
Percentage of tilapia (*O. niloticus*) organs culture positive for *S. agalactiae* following inoculation at two doses and freezing for 7, 14, 30, and 180 days at −70 °C

S. agalactiae dose[a] CFU/fish	Organ sampled	Fish *N*[b]	Frozen storage period[c]			
			7 Days	14 Days	30 Days	180 Days
5.5×10^2	Nare	5	100%	100%	100%	100%
5.5×10^2	Brain	5	100%	100%	100%	100%
5.5×10^2	Intestine	5	80%	100%	100%	80%
5.5×10^2	Head kidney	5	100%	100%	100%	100%
4.5×10^6	Nare	5	100%	100%	100%	100%
4.5×10^6	Brain	5	100%	100%	100%	100%
4.5×10^6	Intestine	5	100%	80%	100%	80%
4.5×10^6	Head kidney	5	100%	100%	100%	100%

[a] Tilapia IP inoculated with 5.52×10^2 CFU/fish and 4.52×10^6 CFU/fish of *S. agalactiae*. Control ($N=20$) fish received 0.1 ml tryptic soy broth (TSB). *S. agalactiae* was not isolated from any organs of the control fish at Day 0 or after any time period post-freezing.
[b] Number of fish organs swabbed and cultured per time period.
[c] Length of time fish were kept frozen at −70 °C.

yielded viable *S. agalactiae* (Table 1). Ninety-five percent of mullet intestine were culture positive for *S. agalactiae*. The culturing of previously un-sampled organs from culture positive mullet resulted in an overall increase in the percentage of culture positive organs from frozen mullet (98.7%) (Table 1).

3.2. Experimental tilapia: mortality and S. agalactiae retrieval

Thirty percent of the fish were dead at each injection dose within 24 h. Both mortality and morbidity of tilapia infected at 4.5×10^6 and 5.5×10^2 CFU/fish were 50% and 40%, respectively. The percentage of asymptomatic fish was 50% for fish injected with 4.5×10^6 CFU/fish and 60% for fish injected with 5.5×10^2 CFU/fish. No control fish showed disease signs or died. Nare, brain, intestine, and head kidney were culture negative for *S. agalactiae* from all control fish at 0, 7, 14, 30, and 180 days post-freezing. One hundred percent of tilapia nare, brain, and head kidney were culture positive for *S. agalactiae* at both 5.5×10^2 and 4.5×10^6 CFU/fish doses and all frozen storage periods (Table 2). *S. agalactiae* was recovered from 100% of frozen tilapia intestines following infection at 5.5×10^2 CFU/fish and 14 and 30 days post-freezing, and following infection at 4.5×10^6 CFU/fish and 7 and 30 days post-freezing. All organs were culture positive for *S. agalactiae* following infection at both doses and after freezing for 30 days.

4. Discussion

Viable *S. agalactiae* cultures can be retrieved from both naturally and experimentally infected fish tissues frozen at −70 °C for 9 and 5 months, respectively, after the enrichment technique. Furthermore, detection of bacteria from fish injected with low numbers of

bacteria (5.5×10^2 CFU/fish) indicates that *S. agalactiae* retrieval and viability is not hampered by extended periods of freezing and that this technique may have the potential to detect low levels of infection. In experimental infectivity studies involving viability of various species of Gram negative bacteria in fish frozen at -20 °C, Brady and Vinitnantharat (1990) reported poor longevity of *Aeromonas hydrophila* as compared to other aeromonads, *Pseudomonas fluorescens* and *Edwardsiella* spp., using inocula ranging from 10^6 to 10^8. The survival of *P. fluorescens*, *Edwardsiella* spp., and *A. hydrophila* was 60, 30–50, and 20 days, respectively. Freezing fish at -70 °C and the enrichment technique may have improved the survival and recovery of our Gram positive isolates.

The re-sampling of frozen mullet tissues validated initial isolation of *S. agalactiae* since fresh organs that were culture positive initially remained culture positive when re-sampled. Archived frozen fish allowed us to evaluate increased numbers of fish organs not possible in the field due to time constraints. Retrospective analyses of frozen organs not previously sampled from naturally infected mullet showed an increase in the distribution of *S. agalactiae* in multiple organs of culture positive fish. Two organs from mullet, nares and intestines, not routinely sampled from diseased fish, were 100% and 95% culture positive, respectively. Evans et al., (2001) reported experimental *S. iniae* infection and rapid dissemination in hybrid striped bass (*Morone chrysops* × *Morone saxatilis*) following nare inoculation at a dose of 4.8×10^3 CFU/fish. *Streptococcus iniae* was shown to rapidly disseminate from the nare to brain and internal organs. Isolation of *S. agalactiae* from the nares of tilapia experimentally inoculated at 5.5×10^2 CFU/fish suggests that isolation from the nares is possible following a low injectable dose of *S. agalactiae*. The isolation of *S. agalactiae* from the nares of both fresh and frozen fish indicates that the nares offer a non-invasive site for the detection of streptococcal infection.

Recovery and retrieval of *S. agalactiae* from frozen fish in this study indicates that frozen fish are a viable alternative to microbiological sampling of moribund fish in the field when trained personnel are unavailable. Consideration, however, should be given to behavioral observations during a suspected disease outbreak as this information is vital to a disease investigation and is not apparent from frozen fish. The isolation of *S. agalactiae* was excellent from mullet that were alive and moribund prior to freezing, but we do not know if a loss of viability would have occurred in mullet collected dead and frozen. However, we were able to recover *S. agalactiae* from fresh dead tilapia after extended periods of freezing. In viral studies on the survival of largemouth bass iridovirus in frozen visceral tissues following 155 days post-freezing, Plumb and Zilberg (1999) discovered more rapid virus loss in fish frozen dead than in fish frozen alive. During epidemiological investigations, the use of frozen fish can be an effective method of collection and transport of pathogenic *Streptococcus* spp. until isolation by the diagnostic laboratory. Furthermore, archived frozen fish can be used for retrospective analyses of algal toxins (Glibert et al., 2002) or bacterial recovery from multiple or different tissues.

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